AGPAT Enzyme Activity and Localization in GPAT3 and GPAT4 Knockout Mice

By
Liliya Veliko

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University of North Carolina at Chapel Hill

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Approval: Approved
Rosalind Coleman, PI
Bob Bourret, Reader
Saskia Neher, Reader

Abstract:

Triacylglycerol (TAG) is a fat that serves as a vital energy source in eukaryotes. TAG is synthesized *de novo* in the liver and fat cells through a pathway that is initiated by the enzyme glycerol-3-phosphate acyltransferase (GPAT) and next catalyzed by acylglycerolphosphate acyltransferase (AGPAT). It was hypothesized the deletion of GPAT would result in decreased activity in the TAG synthesis pathway and therefore decreased AGPAT activity. To investigate this idea, the activity of AGPAT was assayed in tissue homogenates from liver and adipose of mice lacking GPAT3 or GPAT4, the main GPAT isoforms. Contrary to the hypothesis, AGPAT activity was elevated in GPAT3-deficient and GPAT4-deficient liver samples and adipose samples compared to those prepared from wild type mice. This result suggests the activity of the TAG synthesis pathway increases to compensate for the absence of the rate limiting enzyme, GPAT. In addition to AGPAT's responsiveness to GPAT, the sub-cellular localization of AGPAT was also explored. AGPAT was known to localize to the ER membrane, but it is hypothesized that the ER shares membranes with lipid droplets, the organelles that store TAG. Thus, we predicted that AGPAT activity would be detectable in isolated lipid droplets. AGPAT activity was measured in lipid droplet and microsomal fractions of murine wild type, GPAT3-deficient and GPAT4-deficient livers. AGPAT activity was indeed detected in the lipid droplet fraction, supporting our hypothesis that the ER membranes are contiguous with lipid droplets. AGPAT activity was higher in lipid droplet and microsomal fractions in GPAT3-deficient tissues compared to wild type mice, consistent with our results above and suggesting a general cellular response to the absence of a major GPAT isoform. Understanding the functions and interactions of the enzymes along the TAG synthesis pathway can provide insights that will

inform the development of treatments for metabolic conditions such as heart disease, obesity, and diabetes, in which it is desirable to control TAG synthesis.

Introduction:

Triacylglycerol (TAG) is a vital energy source in every eukaryote. In mammals, TAG is most abundantly produced in specialized cells called adipocytes, which are found in subcutaneous fat deposits, around internal organs, and within muscles [1][2]. Lipid droplets are organelles within cells that store TAGs, influence hormone storage and signaling, and regulate the production and breakdown of fats [3]. Lipid droplets and the enzymes in the glycerolipid pathway are instrumental in regulating fat production and breakdown, yet the interactions between lipid droplets, membrane proteins, and other organelles are not well understood. Metabolic pathologies, such as obesity, cardiovascular disease, and diabetes share a common factor: aberrant fat synthesis or oxidation. Understanding the interactions, movement, and expression of proteins in the TAG biosynthetic pathway could lead to new treatments for heart disease, insulin resistance, and obesity.

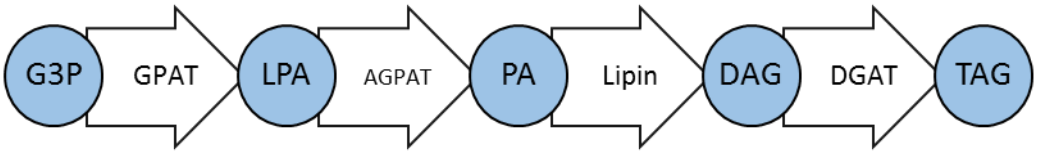

Figure 1: Pathway of de novo TAG production. Circles indicate an intermediate compound and arrows indicate an enzyme [6].

The glycerol-3-phosphate acyltransferase (GPAT) enzyme catalyzes the first step in TAG production: glycerol-3-phosphate (G3P) is esterified to lysophosphatidic acid (LPA). The second step is catalyzed by the acylglycerolphosphate acyltransferase (AGPAT) enzyme,

which acylates LPA to become phosphatidic acid (PA). Lipin removes the phosphate group on PA, leaving a hydroxide group and producing diacylglycerol (DAG). Diglyceride acyltransferase (DGAT) acylates DAG to produce TAG. Two isoforms of the GPAT enzyme, GPAT3 and GPAT4, are located in the endoplasmic reticulum (ER) membrane and account for 50-70% of the GPAT activity in the liver and up to 90% in most other tissues [6]. GPAT enzymes have garnered greater scientific inquiry than AGPAT enzymes because they perform the initial and rate-limiting step of TAG synthesis, leaving AGPAT enzymes comparatively understudied [6]. Eight AGPAT isoforms have been identified, but only the ER membrane-bound AGPAT1 and AGPAT2 have been comprehensively studied [6]. This study examines AGPAT enzyme activity in murine liver in the absence of GPAT3 or GPAT4 to elucidate the relationship between the AGPAT, GPAT3, and GPAT4 enzymes. Because the absence of GPAT4 results in a 45-50% decrease of TAG in murine liver compared to wild type mice [6], it is predicted AGPAT activity will also be decreased in the absence of GPAT4. Since GPAT3 and GPAT4 are both major isoforms of GPAT [4][6], it is also predicted that AGPAT activity will be decreased in GPAT3 knockout murine tissues.

In addition to the relationship of the AGPAT and GPAT enzymes, the possible subcellular localization of AGPAT to fat storage organelles will also be investigated. Absence or mutation of the enzymes in the TAG synthesis pathway may result in abnormal or absent fat production and common metabolic conditions such as insulin resistance and fatty liver disease [4][6]. Surplus lipid storage by the lipid droplets within cells is linked to related metabolic conditions such as obesity and diabetes [3]. The scientific community has regarded lipid droplets as inactive collections of lipids until recently, and therefore their functions as dynamic organelles are not well characterized. The relationship between lipid droplets, their associated proteins,

and their interactions with other organelles are also not well understood [3]. The ER budding model proposes that the ER membrane and integral proteins are incorporated into lipid droplets [3]. To investigate the localization of AGPAT, the enzyme's activity is measured in isolated lipid droplets and isolated microsomes of GPAT3-/-, GPAT4-/-, and wild type mice. Because the major AGPAT isoforms are normally embedded in the ER, detection of AGPAT activity in the isolated lipid droplets would suggest the ER had shared membrane and integral proteins with the lipid droplets. It is predicted AGPAT enzyme activity will be detected in isolated murine lipid droplets, supporting the ER budding model's prediction that lipid droplet and ER membranes are continuous.

Understanding the biological links between lipid droplets, GPAT, and AGPAT could inform treatments for heart disease, diabetes, and obesity. Measuring the activity of GPAT and AGPAT enzymes in each genotype increases understanding of the greater network of these enzymes' interactions and functions. This project will explore AGPAT activity in the liver and adipose tissues, as well as microsomal and lipid droplet fractions, of GPAT3 knockout, GPAT4 knockout and wild type mice.

Methods:

Sample Preparation: Total membrane particulates were prepared from the livers or adipose tissue of mice from each genotype as described by Wendel et al. [5]. Lipid droplet fractions and microsomal fractions were prepared by pooling three mouse livers from mice of each genotype.

BCA Assay: The concentration of protein in each total particulate sample was determined through a bicinchoninic acid assay (BCA) using Pierce™ BCA Protein Assay Kit.

Enzyme Activity Assay: A radiometric enzyme activity assay was used to determine the activity of the AGPAT enzyme in liver and adipose total particulates, or lipid droplet and microsomal fractions of GPAT3-/- and GPAT4-/- mice, as well as wild-type controls. AGPAT activity was calculated by measuring the amount of radiolabeled PA product formed per milligram of protein per minute. The enzyme activity assay for AGPAT was prepared with 119 µL AGPAT mix (100 mM Hepes NaOH pH 7.5, 200 mM NaCl, 5% glycerol, 50 µM 16:0 CoA, 20 µM [^{3}H] oleoyl LPA, 1 mg/mL BSA, 8 mM NaF, 10 mM EDTA, 1 mM DTT). Enough water was added for the final volume of each reaction to be 200 µL. The 16:0 palmitoyl CoA functions as the acyl donor for the conversion of tritium [^{3}H] labeled LPA to [^{3}H]-PA, to be measured and quantified.

Medium I (250 mM sucrose, 10 mM Tris buffer pH 7.5, 1 mM EDTA) was combined with protease and phosphatase inhibitors and was used to dilute the protein sample to 0.25 mg/ml.

The reaction was initiated by the addition of either 5, 10, or 15 µg of protein to the AGPAT mix and proceeded for 5 min. The reaction was stopped with 600 µL of 1% perchloric acid and 3 mL of chloroform: methanol (1:2, v/v).

Lipid Extraction: The samples sat for 5 min at room temperature, and then the phases were broken by the addition of 1 mL of 1% perchloric acid and 1 mL of chloroform. The samples were centrifuged for 5 min at 3,000 rpm at 4°C. Next, the upper phase was aspirated and the remaining solvent was washed with 2 mL 1% perchloric acid. Each sample was vortexed. The samples were centrifuged again with the same settings, and the upper phase was aspirated again. The second wash used 2 mL of 1% perchloric acid. After being vortexed again, the samples were centrifuged for the third time at the same setting. The upper phase was aspirated once more. One mL of the solvent phase containing the extracted lipids was dried in a Speedvac concentrator. After drying, the samples were stored at -20 °C.

Thin Layer Chromatography: Dried lipid extracts were resuspended in 50 µL of 2:1 chloroform: methanol (v/v). A 20-channel silica thin layer chromatography plate was washed with acetone and air dried before spotting. Each resuspended sample was spotted onto a well. Next, 10 µL of PA standard were spotted in each lane (5 mg/ml, 1,2-dioleoyl-G3P, Avanti). The plate was developed using an 88% formic acid: pyridine: chloroform (7:30:50, v/v/v) solvent system. After drying, the plate was sprayed with 0.05 mg/ml primuline and the PA standard was visualized using UV light. Each sample was scraped from the TLC plate into a scintillation vial with 4 mL of EcoLite (MP Biomedicals, scintillation cocktail). Two scintillation vials were prepared with 4 mL of EcoLite and 10 µL of 200 µM [^{3}H]1-oleoyl-LPA to calculate the specific activity. The decay rate of the [^{3}H]-label in each sample was counted using a Wallac L5C 1409 liquid scintillation counter.

Results

AGPAT Activity is Higher in GPAT3 and GPAT4 Knockout Mice Compared to Wild Type

It is known TAG levels are 45-50% lower in the livers of GPAT4 knockout mice compared to wild type mice [6]. It is logical that GPAT activity declines when a major GPAT isoform is absent, but it is not known how AGPAT activity adjusts to the deficiency of the rate limiting enzyme. It was predicted that AGPAT activity would be decreased in GPAT3 and GPAT4 knockout mice compared to wild type. AGPAT enzyme activity was assayed in the liver total particulate and adipose total particulate of wild type, GPAT3 knockout, and GPAT4 knockout mice.

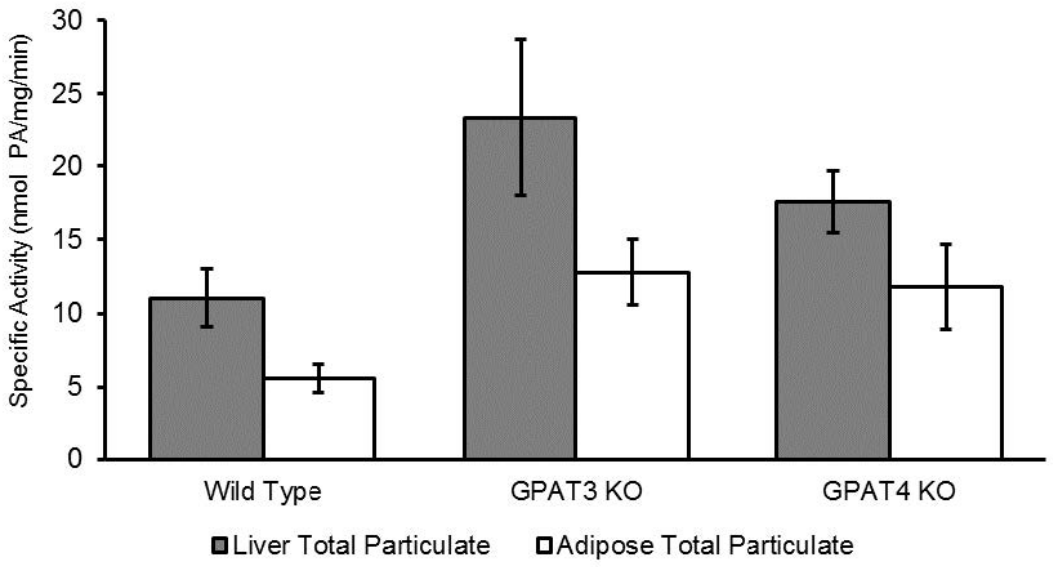

Figure 2: AGPAT activity is higher in the liver and adipose total particulate of either GPAT3 or GPAT4 knockout mice, compared to wild type. For all genotypes, n=3. Error bars represent +/- SD.

Wild type mice showed significantly lower AGPAT enzyme activity in the liver total particulate and adipose total particulate than the knockout mice in the respective tissue samples (Figure 2). In all three genotypes, enzyme activity was lower in the adipose tissue than in liver tissue of the same genotype. AGPAT activity is higher in GPAT3 and GPAT4 knockout murine tissues compared to wild type, in contrast to the prediction.

AGPAT Activity was Detected in Isolated Lipid Droplets, in Agreement with ER Budding Hypothesis

AGPAT was previously found to localize to the ER [6], but it was hypothesized that the ER may share membranes with lipid droplets [3]. In the next experiment, the enzyme activity assay was used to investigate the cellular localization of AGPAT. Differential centrifugation

was used to prepare microsomal and lipid droplet fractions from livers of wild type, GPAT3 knockout and GPAT4 knockout mice (Figure 3).

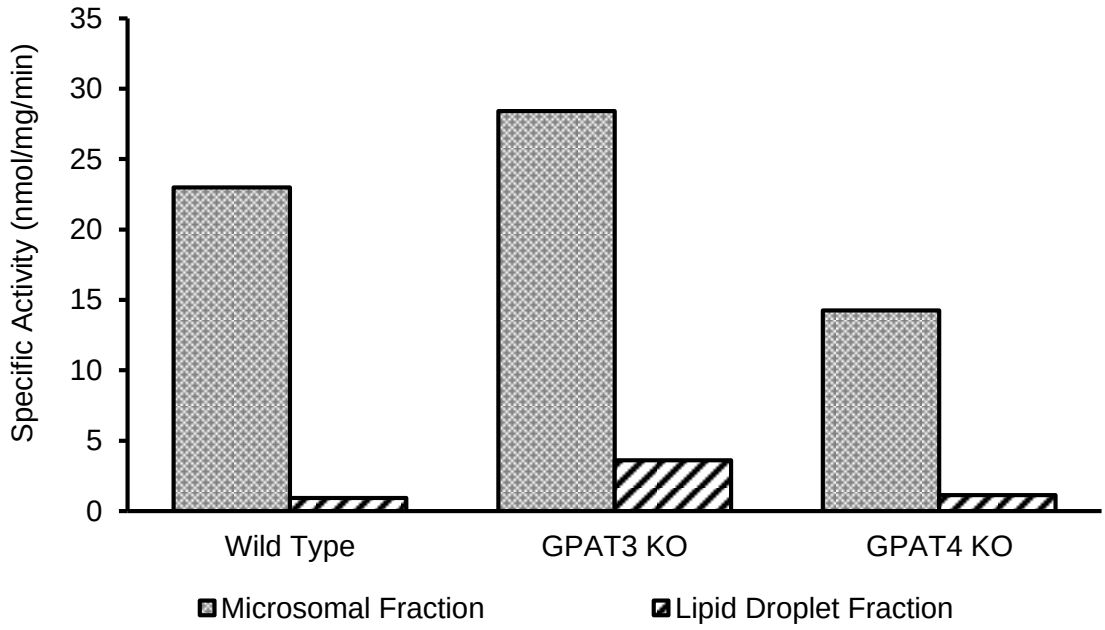

Figure 3: AGPAT enzyme activity was detected in the lipid droplet fractions of wild type, GPAT3 knockout and GPAT4 knockout mice. For each genotype, n=1.

AGPAT enzyme activity was assayed in lipid droplets and microsomes of mice. Fractions were prepared by differential centrifugation of a pool of three mouse livers for each genotype. Specific AGPAT activity was higher in the microsomes than the lipid droplets across all three genotypes. GPAT3 knockout mice had higher microsomal and lipid droplet AGPAT activity than wild type and GPAT4 knockout mice. Lipid droplets from wild type mice had the lowest AGPAT activity. It was predicted that AGPAT activity would be detected in the isolated lipid droplet fraction, suggesting the ER may share membrane and integral proteins with the lipid droplets.

Discussion:

The initial rate enzyme assay measures the quantity of product an enzyme can produce over a given amount of time. The assay provides a reaction environment unlike that of a cell. The enzyme is presented with functionally unlimited substrate in an environment optimized for temperature, pH, and salt content. By definition, in an initial rate enzyme assay, the enzyme converts substrate to product at a maximal rate as a result of the ideal reaction conditions. Consequently, the assay does not measure the absolute activity of the enzyme in vivo, but is a reproducible method of determining relative activity of the target enzyme under the experimental conditions, such as the differing tissue types or mouse models. Since not every AGPAT enzyme will be active during the assay, an elevated specific activity does not denote a higher concentration of AGPAT enzyme, rather a higher rate of PA production by the enzymes present in the sample cells given the experimental conditions.

AGPAT enzyme activity is higher in GPAT3 or GPAT4 knockout mice, both in liver tissue and in adipose tissue, compared to wild type mice (Figure 2). Hepatic TAG levels are 45-50% lower in GPAT4 knockout mice compared to wild type mice [6]. Because the absence of GPAT4 resulted in decreased net TAG production, it was predicted AGPAT activity would also be decreased in GPAT4 knockout mice. Since GPAT3 and GPAT4 together constitute 50-70% of GPAT activity in the liver and 90% in other tissues [4][6], it was predicted that the net TAG production in the absence of GPAT3 may resemble the decreased TAG production in the absence of GPAT4. Therefore, it was also predicted that AGPAT activity would be decreased in GPAT3 knockout mice compared to wild type mice. When assessing AGPAT enzyme activity in GPAT3 knockout mice, it is important to consider that GPAT3 has been reported to have AGPAT activity [7], which may lead to an inaccurately low measurement of AGPAT

activity in its absence. Higher AGPAT activity in GPAT3 knockout and GPAT4 knockout mice compared to wild type mice suggests the overall decrease of TAG in GPAT4 knockout mice is not a direct result of decreased activity in the production pathway. AGPAT activity may be higher in GPAT3 and GPAT4 knockout mice because a feedback mechanism notifies the cell of decreased GPAT activity and attempts to correct for the lower amount of LPA, perhaps, by upregulating AGPAT. It is also possible that in the absence of GPAT3, regulatory mechanisms notify the cell of decreased GPAT activity and so the cell upregulates GPAT4, or another GPAT isoform, to compensate. In GPAT3 knockout mice, it is possible that GPAT4 is compensating and producing more LPA than in wild type mice, and likewise with GPAT3 in GPAT4 knockout mice. A complementary experiment should measure GPAT3 and GPAT4 mRNA transcripts in each knockout mouse to determine relative transcription levels and use western blotting to determine the expression levels of each protein isoform in the absence of the other. GPAT3 and GPAT4 transcripts and protein quantities should also be measured in wild type mice as a control.

Wild type, GPAT3- and GPAT4- knockout mice had higher AGPAT specific activity in microsomes than in lipid droplets (Figure 3). This finding is reasonable considering microsomes are composed of ER and AGPAT is embedded in the ER membrane [6]. GPAT3 knockout mice had the highest AGPAT activity in both lipid droplets and microsomes, while wild type mice had the lowest AGPAT activity in lipid droplets. The observation that GPAT3 knockout mice had higher AGPAT activity in both fractions (Figure 3) supports the ER budding hypothesis, which proposes that the ER membrane and integral proteins are incorporated into lipid droplets [3]. AGPAT, which is embedded in the ER membrane, could be transferred to the lipid droplet with the movement of the ER membrane phospholipids. If membrane sharing

between the ER and the lipid droplets is similar among GPAT3 knockouts, GPAT4 knockouts, and wild type mice, then greater AGPAT activity in the ER membrane would lead to a proportionally greater activity in the lipid droplet. For example, if GPAT3 knockout mice had more active AGPAT in the microsomes, and all genotypes share membrane between the ER and lipid droplets at similar rates, then proportionally more AGPAT enzyme activity would be detectable as the enzyme was sent from the ER to the lipid droplets compared to other genotypes. The AGPAT activity in the lipid droplets of wild type and GPAT4-deficient mice was low but detectable (Figure 3). To agree with the lipid budding hypothesis, one genotype would have to have displayed the lowest AGPAT activity in both fractions. However, wild type mice have the lowest AGPAT expression in lipid droplets while GPAT4 mice have the lowest activity in the microsomes out of the three genotypes. The AGPAT activity in the lipid droplets of wild type and GPAT4 knockout mice are nearly identical and relatively low, so a larger sample size should be assayed to determine if a pattern between relative AGPAT activity in the microsomes and lipid droplets exists.

In contrast to the prediction, AGPAT activity was higher in GPAT3 and GPAT4 knockout mice compared to wild type mice (Figure 2). The higher AGPAT activity suggests a possible cellular response to compensate for the missing GPAT isoform. Western blotting can be used to confirm suspected overproduction of the AGPAT enzyme in GPAT3- and GPAT4-knockout mice. Determining how the TAG synthesis pathway is regulated is instrumental in understanding pathologies such as insulin resistance.

AGPAT activity in lipid droplet fractions implies that the enzyme is present in the lipid droplets of murine liver cells. The correlation between increased AGPAT activity in the lipid droplets and microsomes of GPAT3 knockout mice agrees with the hypothesis that AGPAT

may be transferred from the ER to the lipid droplets by membrane budding. The data garnered in this project is important in making progress toward a full understanding of fat production and regulation. Elucidating the workings of the proteins involved in TAG synthesis is necessary for developing treatments of prevalent ailments, including diabetes, obesity, lipodystrophy, and heart disease.

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